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TITLE: Antibody Induced Perturbation - a New Method to Identify Pathways in Breast Cancer Progression, Invasion and Metastasis

PRINCIPAL INVESTIGATOR: Michael D. Johnson, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20057-1411

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Michael D. Johnson, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Georgetown University Medical Center
Washington, DC 20057-1411

E-Mail: johnsom@georgetown.edu

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**13. ABSTRACT** ***(Maximum 200 Words)***

There is a critical need for markers of metastasis that will allow patients who are node negative to be separated into two groups: those who need further therapy and those who have been cured by their surgery. We hypothesized that changes in cellular biochemistry, that need not involve changes in the expression of particular genes, are important determinants of metastatic behavior, and we further hypothesized that many of these changes might be mimicked by the binding of an antibody with the right binging characteristics. We set out to test the idea that novel determinants of metastatic behavior could be identified by expressing single chain antibodies inside non-metastatic cells, and then screening those cells for the acquisition of a more metastatic phenotype. The antibody responsible for this increased metastatic ability would then be isolated from these cells and used to identify the protein with which it interacted. The project has met with significant technical challenges which have slowed progress. Nevertheless, significant progress has been made to overcome these difficulties and we anticipate being able to complete the study, having been granted a no-cost extension of one year.

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## Introduction:

Patients that die of breast cancer do so principally because of metatsiatic deposits of their disease. Thus, understanding the process of metastasis is an important goal in breast cancer research. There is a critical need for markers of metastasis that will allow patients who are node negative to be separated into two groups: those who need further therapy and those who have been cured by their surgery. Much has been learned about the role of proteases, growth, motility, angiogenic and survival factors, and other molecules involved in metastasis. Much, however, remains to be discovered, particularly about how the normal regulation of the activity of these proteins is disrupted during tumor progression. Attempts have been made in the past to identify new genes associated with metastatic behavior, but all of these attempts have shared the same weakness, in that they have only looked at changes at the level of gene expression. This ignores all the other way that the activity of a protein or biochemical pathway can be altered; changes in phosporylation state, changes in glycosylation, altered conformation, proteolytic cleavage, altered protein stability, altered sub-cellular localization, changes in enzymatic activity, etc.

In this study we hypothesized that changes in cellular biochemistry, that need not involve changes in the expression of particular genes, are important determinants of metastatic behavior, and we further hypothesized that many of these changes might be mimicked by the binding of an antibody with the right binging characteristics.

In this Exploration Award, we proposed to test the idea that novel determinants of metastatic behavior could be identified by expressing single chain antibodies inside non-metastatic cells, and then screening those cells for the acquisition of a more metastatic phenotype. The antibody responsible for this increased metastatic ability would then be isolated from these cells and used to identify the protein with which it interacted.

## Body:

The project was designed to capitalize on the availability of an existing, highly diverse synthetic single chain antibody library. The plan was to transfer the DNA cassettes that codes for the single chain antibodies in this library from the phage display vector, in which the library was constructed, and to place then in a eukaryotic expression vector, thus recapitulating the library in a vector that would allow intracellular expression of the antibodies in cancer cells. This seemingly simple step has proven to be much more technically challenging that we expected and has significantly delayed our progress as will be described below. As a result we have requested, and been granted an extension of the project for an additional year. The vector we proposed to use in this study is a self contained, tetracycline regulable, retroviral construct which, it was planned would allow very efficient transfer of the library into the target cells. Transduced cells were then to be screened by a variety of *in vitro* and *in vivo* assays to identify clones with a more progressed phenotype, after which the causative antibodies were to be isolated and used to purify, identify and characterize the proteins with which they interacted. It was planned that within the first year of the study we would have constructed the vector,

transdced the cells, selected clones, isolated the antibodies and be conducting validation studies on the selected antibodies. The characterization of the antibodies was to be done in the remaining 6 months of the project. With hindsight, this was an ambitions schedule even without any technical difficulties.

**Progress to date:**

Two general strategies were proposed by which the antibody cassette was to be transferred from the phage vector to the retroviral vector: 1) PCR based amplification of the cassette and 2) a restriction digestion based sub cloning method. The problem is that in order for the project to have any chance of working, it is essential that the diversity of the antibody library be maintained at the highest level possible. Thus, the transfer has to be done in such a way that the representation of the library be maintained. This has proved to be very difficult.

We initially thought that the PCR based approach was going to be the easiest and most likely to maintain diversity. However, the library was originally constructed by a sub-cloning strategy using sub-cassettes flanked by restriction sites for rare, 8 base cutting enzymes. These sequences, therefore, contain fairly long runs of poly-C or –G which made designing PCR primers that would a) produce adequate amplification, b) maintain the appropriate reading frame of the cassette to be transferred, c) minimize the inherent bias of a pool based PCR reaction and d) allow directional insertion into the retroviral vector very difficult. We have spent a considerable amount of time refining the design of these primers, optimizing the amplification strategy and testing a variety of polymerases that allow us to use conditions that minimize the effects of the very GC rich primers we have to use. During this process it became apparent that we needed methods that would allow us to evaluate the diversity of subsections of the library so that we could optimize our approach. This we did by a sampling-sequencing based approach and we discovered that amplification conditions had a very significant effect on the ultimate diversity of the pooled amplification products. This has again led us to re-design our strategy.

In parallel we were developing a conventional restriction enzyme based subcloing approach, which progressed more smoothly. The down-side of this approach is that we can only use the enzymes used to generate the original library without risking the loss of diversity due to unintended digestion at sites within the cassette. This limits flexibility and has been a problem since, as described below; we have had to work with a different vector than we had planned to. Nevertheless, this approach has worked quite well and we have shown that we can transfer the library without damaging the ultimate diversity significantly.

In the proposal we had planned to use the retroviral vector pBSTR1. We have subsequently discovered, however, that this vector is not suitable for this study. We had previously used this construct in other projects and it had performed well. It rapidly became apparent as we started to use it in this project that there were several issues with the construct that would be a problem. This most significant one is that it is quite difficult to reliably obtain high titer viral stocks. This is a problem since we need

extremely high titer stocks in order for the screening assays to adequately sample the diversity of the library. The issue of the adequacy of the screens ability to sample the full diversity of the library has always been the weak link of the project as we acknowledged in the initial proposal, and low titer viral stocks is an unacceptable additional problem. We have, therefore, examined alternate approaches. One is to use a binary tetracycline system and we have made progress developing the cell lines for use in this approach, transfecting several cell lines with the transactivator protein in preparation for their use in this context. In an alternate approach we have evaluated another self-contained retroviral vector – pLRT (PubMed ID 9175791). We have shown that we can obtain very high titer stocks using this system and that the construct overcomes another problem that we were experiencing with pBSTR1, that of poor tetracycline regulability, and poor slectability. The organization of the transcription cassettes in pBSTR1 is such that they interfere with each other in certain contexts. This causes problems with the efficacy of the selection marker and with the efficiency of tetracycline regulation. The new vector we are using gets around these problems since the cassettes are differently arranged and the vector makes use of a much cleaner selection marker – blasticidin rather than puromycin. This will greatly simplify the selection of transduced clones once we start that part of the project.

There is, however a down side to the use of this construct. The available restriction sites for the insertion of the gene to be expressed (in this case the synthetic antibody cassette) are extremely limited and not very convenient with respect to the constraints of the system we are using. This is not much of a problem for the more conventional use of the vector – to express one gene of interest, since a couple of simple steps allow almost any insert to be cloned into the vector. This is not a practicable approach for the insertion of the antibody cassettes, firstly since we are very limited in the enzymes that we can use, and secondly since any additional step is extremely undesirable due to the deleterious effect of any additional step on the diversity of the ultimate library. This is one reason we are still aggressively pursuing the PCR based strategy since this allows us much more freedom in how we work with the cassette, and we are also developing a modified version of pLTR that will simplify the facile directional insertion of the library. We believe we are very close to having several versions of the library in usable forms.

While we have been working through the problems with the manipulation of the library we have been evaluating and conducting preliminary studies with the screens we will use with the libraries in their final form. As noted above, the issue of the abilities of the screens to adequately evaluate the true diversity of the libraries has always been a tricky issue. We have developed a series of pre-screens that we believe will allow us to maximize library coverage to try and overcome this issue. All of the materials and methodologies are developed and ready to go once the library development is complete.

With the additional time that has been granted in the form of a no-cost extension for one additional year, we remain confident that we can complete the work substantially as planned, with the noted substitution of reagents. The goals of the study remain unchanged as does our commitment to the project.

Key Research Accomplishments:

1) We have developed methods to overcome the technical challenges involved in sub-cloning the antibody cassette while maintaining the diversity of the library.

2) We have developed a novel self-contained retroviral vector variant to facilitate the work.

3) We have refined the screens that will be used to identify components of the library that impart a more progressed phenotype on transduced cells.

Reportable Outcomes:

None

Conclusions:

We have encountered significant technical challenges in the initial stages of this project which have dramatically slowed progress. However, we have been making steady progress, both towards overcoming these challenges and with respect to developing the screens that we will use once the libraries are ready for use. We remain confident that the work can be completed with the additional time now available due to a no-cost extension of the project for one year.